

CHEMISTRY

A European Journal

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Accepted Article

Title: The Coordination Chemistry of Copper Uptake and Storage for Methane Oxidation

Authors: Christopher Dennison

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To be cited as: *Chem. Eur. J.* 10.1002/chem.201803444

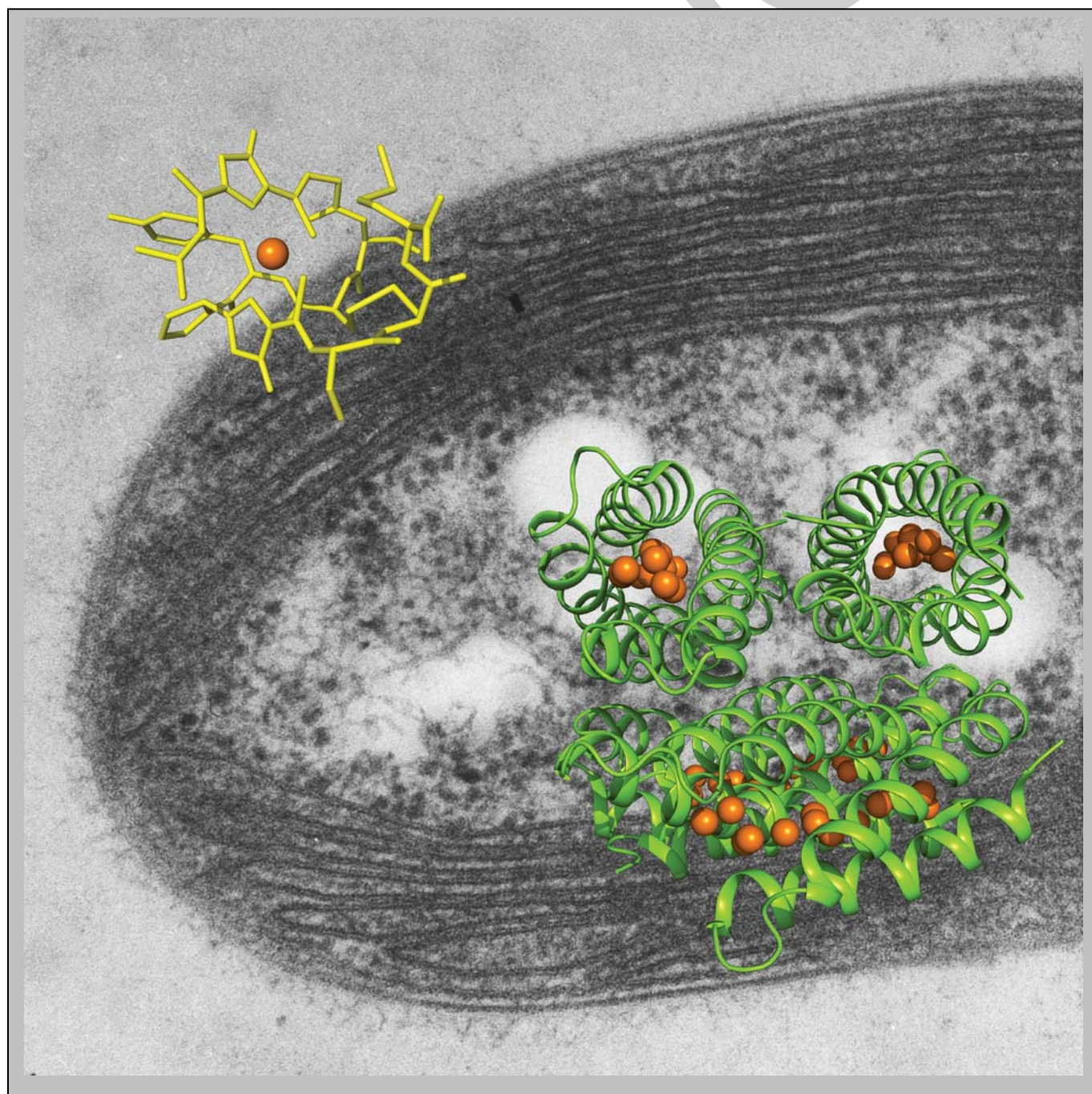
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The Coordination Chemistry of Copper Uptake and Storage for Methane Oxidation

Christopher Dennison*



Abstract: Methanotrophs are remarkable bacteria that utilise large quantities of copper (Cu) to oxidize the potent greenhouse gas methane. To help provide the Cu they require for this process some methanotrophs can secrete the Cu-sequestering modified peptide methanobactin. These small molecules bind Cu(I) with very high affinity and crystal structures have provided insight into why this is the case, and also how the metal ion may be released within the cell. A much greater proportion of methanotrophs possess a member of a newly discovered bacterial family of copper storage proteins (the Csp). These are tetramers of four-helix bundles whose cores are lined with Cys residues enabling the binding of large numbers of Cu(I) ions. In methanotrophs, a Csp exported from the cytosol stores Cu(I) for the active site of the ubiquitous enzyme that catalyses the oxidation of methane. The presence of cytosolic Csp, not only in methanotrophs but in a wide range of bacteria, challenges the dogma that these organisms have no requirement for Cu in this location. The properties of the Csp, with an emphasis on Cu(I) binding and the structures of the sites formed, are the main focus of this review.

1. Introduction to copper in biological systems

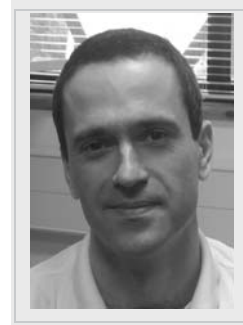
Copper is an essential element for most organisms being required as the cofactor for enzymes in a number of key biological processes.^[1,2] The facile redox reactivity of this metal ion is the main chemical property exploited by Cu enzymes using a range of active site structures. In some cases the sole purpose of the Cu site is to use the interconversion between the Cu(II) and Cu(I) forms to transfer electrons, such as in proteins involved in photosynthesis and respiration. Other sites couple the redox chemistry of Cu to the catalytic conversion of a substrate, including superoxide radicals, phenols, lysine and iron. As with many biologically useful metals, Cu can be toxic when not correctly regulated, and this typically becomes more of an issue at elevated levels when the metal ion is present in excess. Eukaryotes have well-understood homeostasis networks for handling Cu, ensuring safe import into cells, transport to desired destinations and storage when necessary.^[1,2] Some related systems have been identified in bacteria.^[2] However, a prevailing view is that bacteria have evolved to limit their use of Cu to help prevent the potential toxicity of this metal ion. Methanotrophs are an atypical family of bacteria as they can handle large quantities of Cu, which they use at the active site of the main enzyme that oxidizes the potent greenhouse gas methane.^[3] These organisms are therefore ideal for investigating Cu-handling, providing insight into systems and mechanisms that may also be used by non-methanotrophic bacteria.

2. Methanotrophs, methane, and copper

Aerobic methanotrophs are an important group of Gram negative bacteria that can grow using methane as their sole source of carbon and energy, and are widespread in environmental locations where oxygen and methane are present.^[3,4] Almost all methanotrophs need Cu to catalyse methane oxidation using a particulate (membrane-bound) methane monooxygenase (pMMO), which is housed on specialized intracytoplasmic membranes.^[3,5] Some methanotrophs possess a soluble iron (Fe) MMO (sMMO).^[3,5,7] Methane is a potent greenhouse gas produced from both natural and anthropogenic sources, and decreasing levels in the atmosphere is of great environmental importance,^[8-10] with MMOs being nature's primary mechanism for mitigating its release. Therefore, if the ability of methanotrophs, and their MMOs, to metabolise such a plentiful and renewable, yet potentially damaging, carbon source could be harnessed and manipulated, a larger range of biotechnological applications would be possible with these organisms than is currently the case.^[9,10]

A dinuclear Fe site catalyses the oxidation of methane in sMMO.^[11] However, the metal-binding properties of pMMO have been the subject of debate for many years (for example see refs. 7,12-19). This has not only revolved around the number of Cu ions associated with the protein, but also the suggestion that pMMO binds Fe.^[13,14,17] The first crystal structure of a pMMO, from *Methylococcus capsulatus* Bath, was published in 2005 showing the pmoA (α), pmoB (β) and pmoC (γ) subunits arranged as an $\alpha_3\beta_3\gamma_3$ tetramer.^[16] Two Cu sites, one suggested to be mononuclear and the other dinuclear, were located in pmoB, with a zinc (Zn) site at the interface of pmoA and pmoC. The soluble domain of pmoB, housing the purported dinuclear Cu site, was found to bind 1.6 equiv of Cu and have methane-oxidation activity, indicating this as the active site of the enzyme.^[6] In subsequent crystal structures of pMMOs from a range of methanotrophs, variations in the metal sites have been

Chris Dennison received his PhD in Bioinorganic Chemistry from Newcastle University (1994) and then worked as a postdoctoral fellow at Leiden University. He was appointed to the Faculty at University College Dublin in 1997, returning to Newcastle University in 1999 as a Lecturer in Inorganic Chemistry. In 2004 he became a Senior Lecturer in the Institute for Cell and Molecular Biosciences (Medical School) and Professor of Biological Chemistry in 2010. He studies the role of metals, and particularly Cu, in biological systems, which has included the analysis of electron-transfer proteins and multi-Cu redox enzymes. More recently, work has focussed on Cu homeostasis in a range of organisms, including understanding how methane-oxidizing bacteria acquire, handle and store Cu.



[a] Prof. C. Dennison
Institute for Cell and Molecular Biosciences
Newcastle University
Newcastle upon Tyne, NE2 4HH (UK)
E-mail: christopher.dennison@ncl.ac.uk

observed.^[7] Refinement enhancement of crystallography data using quantum-mechanical calculations has very recently suggested that the active site of pMMO most likely consists of a single Cu ion bound by three His residues.^[19]

When methanotrophs utilise pMMO to oxidize methane it can constitute up to ~20% of total cellular protein,^[17] resulting in a large requirement for Cu. In the subset of methanotrophs that also have an sMMO, this enzyme is only expressed when Cu is limiting, with the 'switchover' mechanism regulated by Cu.^[3,5] Some methanotrophs make and secrete modified peptides called methanobactins (Mbns), also when Cu levels are low.^[3,20] These molecules bind environmental Cu with high affinity,^[21-23] and can extract the metal from minerals,^[24] with Cu-Mbn re-incorporated.^[22,25] Other methods have to be present to provide the Cu methanotrophs require, but these have received much less attention.^[25] The latest development demonstrating the fundamental importance of Cu to methanotroph metabolism is the presence of dedicated storage proteins for this metal ion,^[26] which are the main subject of this review. Before discussing these, work on Mbns that ultimately led to the discovery of this novel family of Cu proteins will be summarized.

3. Structure and function of methanobactins

Methanobactins are ribosomally synthesised post-translationally modified peptides that methanotrophs can secrete.^[3,27,28] They exhibit similarities to certain peptidic siderophores,^[29] the best characterized bacterial family of metal-sequestering small molecules. Siderophores were initially thought to be specific for Fe, but an ability to bind other metal ions is emerging.^[30-32] The currently identified Mbn operon is found in approximately 10% of methanotrophs whose genomes have been sequenced,^[33] and is also present in non-methanotrophic bacteria.^[3,27,34] Methanotrophs may well produce other, currently unidentified, Cu-sequestering compounds, and the number of bacterial natural products/small molecules that can bind metal ions, including Cu, is increasing.^[31,32,35,36] The *mbnA* gene codes for leader (cleaved in Mbn) and core peptides, with the latter modified by enzymes also included in the Mbn operon.^[3,34,37-40] Methanobactins from only six methanotrophs have been characterized, purified from media in which the organisms were grown under Cu-limiting conditions.^[20-23,27,41] Methanobactins have a strong preference for binding Cu(I), and the addition of Cu(II) to an apo-Mbn results in the rapid formation of the Cu(I) form,^[21,23] although the mechanism of reduction is not known. For those Mbns whose Cu(I) affinities have been measured, values in the 10^{20} - 10^{21} M⁻¹ range are found, some of the highest known for biological Cu sites.^[21-23] Due to their susceptibility to reduction, the stability constants of Cu(II)-Mbns have been calculated from the reduction potential (E_m value) of a Cu-Mbn, along with its Cu(I) affinity. These are 6-10 orders of magnitude weaker than those for Cu(I).^[21-23]

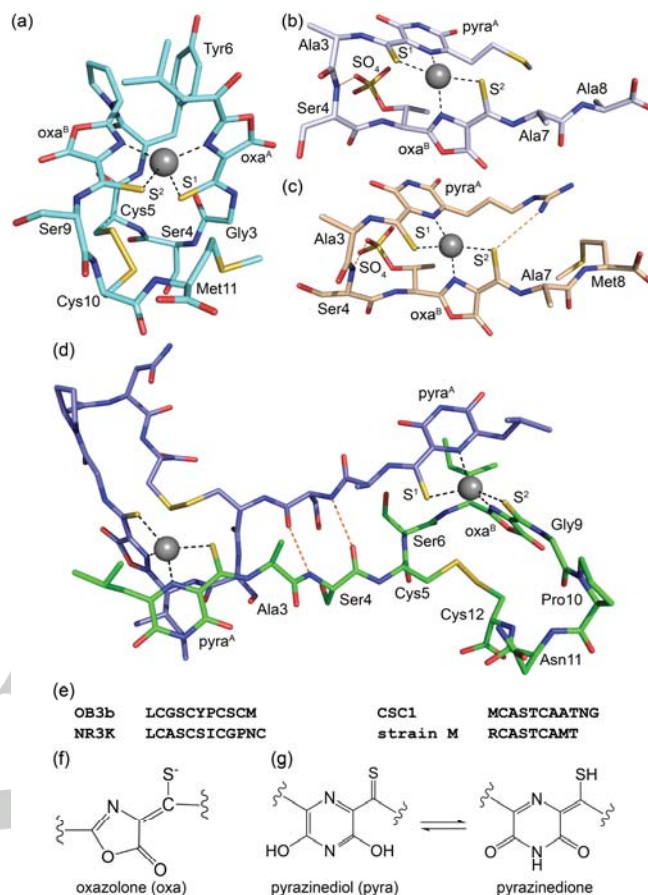


Figure 1. The structures of Mbns. Crystal structures of *M. trichosporium* OB3b (a, PDB file 2XJH), *Methylocystis hirsuta* CSC1 (b, PDB file 2YGI), *Methylocystis* strain M (c, PDB file 2YGJ), and *M. sporium* NR3K (d, PDB file 4OZ7) Cu(I)-Mbns.^[21-23] The *M. sporium* NR3K Cu(I)₂Mbn₂ dimer (d) is made from two symmetry-related monomers that are coloured green and slate. Cu(I) ions are shown as grey spheres and Cu(I)-ligand and key hydrogen bonds as dashed black and orange lines respectively. Amino acid residues not altered by post-translational modifications are numbered according to their position in the core Mbn peptides, whose sequences are shown in (e). The genome of *Methylocystis* strain M has not been sequenced and the incomplete amino acid sequence of its Mbn has been obtained from studies on the peptide.^[22] *M. trichosporium* OB3b Cu(I)-Mbn has two coordinating oxazolone rings (oxa^A and oxa^B), whilst the N-terminal oxa^A is replaced by a pyrazinediol (pyra^A) in all other Cu(I)-Mbn structures. The sulfur ligand provided by the N-terminal thioamide/enethiol group is labelled S¹, and the other S². All of the ligating atoms come from the main modified regions of Mbns, and these are shown as chemical structures in (f) and (g). Tautomeric forms of the six-membered ring other than a pyrazinediol, such as a hydroxypyrazinedione or pyrazinedione (f), would require the non-coordinating nitrogen to be protonated. Careful analysis of high-resolution (0.80 Å) diffraction data for the two *Methylocystis* Mbn structures^[22] shows almost no evidence of a proton at this position consistent with the pyrazinediol form. Crystallized *M. hirsuta* CSC1 Cu(I)-Mbn is missing three C-terminal residues (Thr9, Asn10, and Gly11),^[22,39] whilst that from *Methylocystis* strain M has at least Thr9 absent.^[22] The *Methylocystis* Cu(I)-Mbns lack a disulfide bond, have a sulfate group and possess an overall hairpin-like structure compared to the more compact arrangement of *M. trichosporium* OB3b Cu(I)-Mbn. The Cu(I) sites are remarkably similar in all of the Cu(I)-Mbn structures.

The first Mbn to be characterized was that from *M. trichosporium* OB3b,^[20] whose initial crystal structure was amended on the basis of NMR studies.^[42] The corrections made were confirmed by very high resolution crystal structures of the full length form (Figure 1a), and a derivative produced by the methanotroph without the C-terminal Met residue.^[21] The crystal structures of only three other Mbns are currently available: two from *Methylocystis* strains, and the molecule from *Methylosinus sporium* NR3K (Figure 1b-d).^[22,23] In all cases Cu(I) is bound in very similar N₂S₂ distorted tetrahedral sites; ligands and a geometry that strongly favour Cu(I) over Cu(II). Post-translational processing of Mbns (Figure 1e-g) is key for Cu binding as all of the ligands are from modified regions of the molecule (Figure 1a-d). The coordinating nitrogen atoms are provided by either five- or six-membered heterocyclic rings, with the sulfur ligands originating from thioamides/enethiols (Figure 1). Each coordinating nitrogen is three bonds from a sulfur ligand and two five-membered chelate rings form upon metal binding, contributing to the high Cu affinities. One of the N/S chelating units is very close to the N-terminus, with the second approximately midway along the peptide (Figure 1a-d). In *M. trichosporium* OB3b Mbn both nitrogen ligands are provided by an oxazolone ring (Figure 1a, f).^[21,42] However, in all other Mbn crystal structures the N-terminal chelating unit contains a nitrogen from a pyradinediol ring (Figure 1b-d, g).^[22,23] The sequence variations that exist between Mbns influence their ability to provide Cu to a methanotroph, with the native Cu(I)-Mbn being the preferred source of the metal.^[22]

The high affinities of Mbns for Cu, which promote binding and prevent unwanted release of this potentially cytotoxic metal ion, must provide a challenge when the methanotroph needs to acquire the bound Cu(I). The available E_m values of Cu-Mbns range from ~480 to ~750 mV versus the normal hydrogen electrode (at 25 °C).^[21-23] We previously suggested that oxidation-assisted removal may occur,^[22] particularly in methanotrophs whose Mbns have lower E_m values, such as that from *Methylocystis hirsuta* CSC1.^[22] However, the E_m value of this Mbn (527 mV) along with its tight Cu(I) affinity results in a Cu(II) affinity of $5 \times 10^{14} \text{ M}^{-1}$. Therefore, even if a suitable cellular oxidant exists, a partner with the ability to bind Cu(II) tightly would be required to extract the metal ion. The crystal structure of the Mbn from *M. sporium* NR3K (Figure 1d) has provided the first insight into a mechanism whereby both the thermodynamic and kinetic barriers to Cu(I) removal can be overcome.^[23] The *in vitro* properties of *M. sporium* NR3K Mbn are analogous to those of *M. trichosporium* OB3b, indicating it predominantly exists in solution, particularly at lower concentrations, as a similar Cu(I)-Mbn monomer (Figure 1a). However, in the crystal structure a Cu(I)₂-Mbn₂ dimer is observed (Figure 1d). For this to form the N-terminal region of the molecule has undergone a conformational change, including dissociation of the first N/S chelating unit, resulting in inter-monomer Cu(I) sites, probably stabilized by the high Cu(I)-Mbn concentrations used for crystallization.^[23] It is therefore possible that upon interaction with an appropriate partner in a cell a similar conformational change would enable access to Mbn-bound Cu(I), with removal taking place via ligand exchange. Cu(I) can rapidly equilibrate

between Mbn and the ligand bathocuproine disulfonate (BCS),^[21-23] and different Mbns are able to exchange Cu(I) in solution.^[22] Considering the high Cu(I) affinities of the species involved in these experiments, Cu(I) transfer has to occur via transient ternary complexes, consistent with the proposed mechanism for Cu(I) removal in a cell.^[23]

4. Bacterial copper storage proteins

4.1 The discovery of novel bacterial copper storage proteins

To investigate the cellular fate of Cu(I)-Mbn, attempts were made to identify the molecule in soluble extracts from *M. trichosporium* OB3b using a metalloproteomic approach.^[26] The Cu-peptide, and also the apo form, were not found, but a number of soluble Cu-containing fractions were observed. In the most abundant of these, Cu was associated with a previously uncharacterized protein.^[26] *In vitro* studies showed this to be a tetramer of four-helix bundles with 13 Cys residues distributed along, and all pointing into, their cores (Figure 2a). Each monomer has 13 Cu(I) sites, and a fully-loaded tetramer can therefore accommodate 52 Cu(I) ions, consistent with a role in storing cuprous ions. This initial member of a new family of Cu storage proteins, the Csp, was called Csp1 (*MtCsp1* as it originates from *M. trichosporium* OB3b).^[26] It has a predicted twin arginine translocase (Tat) signal peptide, consistent with folding prior to export from the cytosol,^[43] potentially with Cu(I) ions bound, which it stores for, and may also deliver to, pMMO.^[26,33] Homologues with a predicted Tat signal peptide (*MtCsp2*) and without a signal peptide (*MtCsp3*) are present in *M. trichosporium* OB3b,^[26] but the specific functions of these remain unknown.^[33] *MtCsp3* shows greater sequence variation to *MtCsp1* with only 26% identity, compared to ~60% between *MtCsp1* and *MtCsp2*, and has 18 Cys residues. Homologues of *MtCsp1* and *MtCsp3* exist in over 40% of methanotrophs whose genomes are available, with Csp3s more abundant, and are thus much more common than the Mbn operon (present in ~10% of these organisms, see Section 3).^[33] Cytosolic Csp3s are also more widespread and abundant than exported Csp1s in non-methanotrophic bacteria, and are found in some Archaea. This is somewhat surprising and challenges the idea that bacteria do not use Cu in this location to help minimize its potential toxicity. *In vitro* characterization of *MtCsp3* and the homologues from *Bacillus subtilis* and *Streptomyces lividans* (the latter almost 50% identical to *MtCsp3*), which have 18 and 19 Cys residues respectively, show they also form tetramers of four-helix bundles, but are capable of binding more, up to ~80, Cu(I) ions (Figure 3a).^[44,45] The Cu(I) capacity of a Csp is directly related to the number of Cys residues it possesses, and cytosolic Csp3s generally have more than the exported Csp1s.

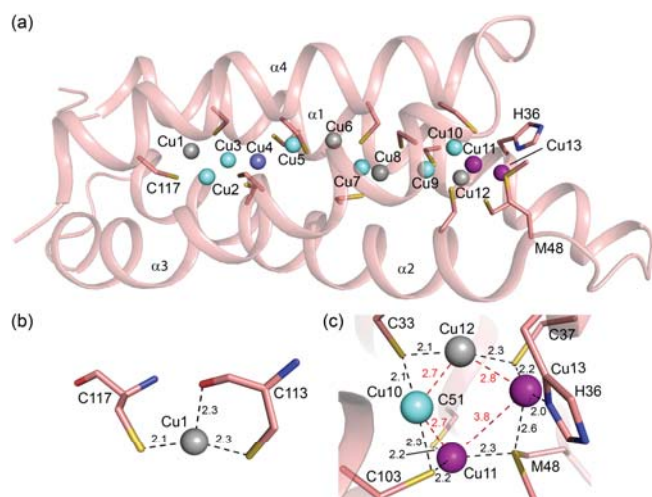


Figure 2. The crystal structure of *MtCsp1* binding 13 equiv of Cu(I).^[26] (a) The four-helix bundle of a Cu(I)-*MtCsp1* monomer (PDB file 5FJE, chain A) with Cu(I) ions as light grey (group 1), aquamarine (group 2), and purple (group 3) spheres (Cu4 is a group 2 site coordinated by three Cys residues from different α -helices and is shown as a slate sphere). The side chains of all Cys residues (three, two, three and five on α_1 , α_2 , α_3 and α_4 , respectively), His36 and Met48 are represented as sticks and the α -helices are numbered. (b) A typical group 1 site (Cu1) coordinated by the C¹¹³XXXC¹¹⁷ motif, showing the close approach of the backbone carbonyl oxygen of Cys113 to the Cu(I) ion. (c) The detailed structure of the four Cu(I) sites at the mouth of the Cu(I)-*MtCsp1* bundle. Cu(I)-ligand bonds and Cu(I)-Cu(I) interactions are shown as black and red dashed lines, respectively, with distances in Å.

4.2 Structures of the Cu(I) cores of copper storage proteins

In their crystal structures the cores of Cu(I)-*MtCsp1* (Figure 2a) and Cu(I)-*MtCsp3* (Figure 3a) are filled with 13 and 19 Cu(I) ions respectively, and contain novel coordination sites for the cuprous ion in biological systems.^[26,44] Regardless of the common occurrence of four-helix bundles in metalloproteins,^[11,26,46,47] including forming the coat of ferritins that can store large numbers of Fe(III) ions,^[48] and its extensive use in metal site modelling/engineering studies,^[47,49-51] filling the entire core of this fold with metal ions had not been observed previously. In both Cu(I)-*MtCsp1* and Cu(I)-*MtCsp3* the side chains of all Cys residues (13 and 18 respectively) are the major ligands, with other amino acids also involved in coordination (Figure 2a and Figure 3a).^[26,44] The resulting Cu(I) sites can be classified into three groups based on differences in their coordination environment: (1) those bound by two Cys residues on the same α -helix; (2) Cu(I) ions coordinated by two, and in very rare cases three, Cys residues from different α -helices; and (3) Cu(I) sites bound by Cys and other residues. Group 1 and 2 sites form the majority of the Cu(I) cores, whereas group 3 sites are usually found at the mouths of the four-helix bundles via which Cu(I) ions are thought to enter and leave the Csps (Figure 2a and Figure 3a). Access to the core and Cys residues at the opposite ends of the bundles is prevented by hydrophobic side chains.^[26,44]

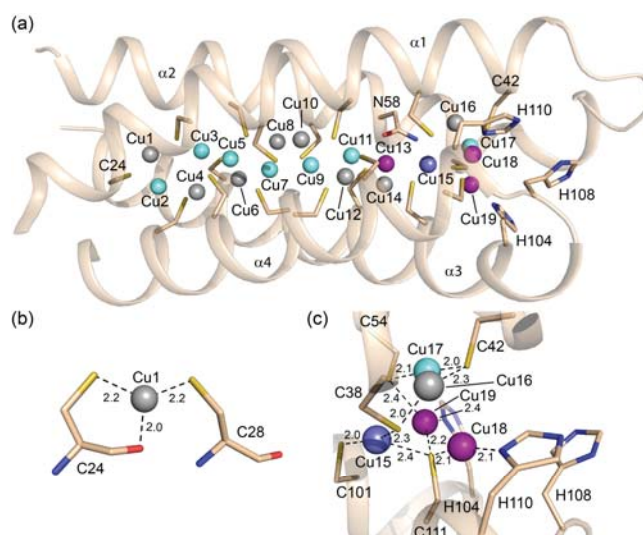


Figure 3. The crystal structure of *MtCsp3* binding 19 equiv of Cu(I).^[44] (a) The four-helix bundle of a *MtCsp3* monomer (PDB file 5ARN) with the N-terminal helix (α_N) omitted, and with spheres representing Cu(I) ions coloured as described in the legend to Figure 2 (Cu15 is a group 2 site coordinated by three Cys residues from different α -helices and is shown as a slate sphere). The side chains of all Cys residues (six, three, four and five respectively on α_1 , α_2 , α_3 and α_4), Asn47, His104, His108 and His110 are represented as sticks and the α -helices are numbered. (b) Coordination at Cu1, a typical group 1 site bound by the C²⁴XXXC²⁸ motif, showing the close approach of the backbone carbonyl oxygen of Cys24 to the Cu(I) ion. (c) The detailed structure of the five Cu(I) sites at the mouth of the Cu(I)-*MtCsp3* bundle with the only significant Cu(I)-Cu(I) interactions between Cu16 and Cu17 (2.8 Å), Cu16 and Cu18 (2.7 Å) and Cu17 and Cu19 (2.5 Å). Cu(I)-ligand bonds are shown as black dashed lines, with distances in Å.

For the Cu(I) sites of Csps coordinated by two thiolates on the same helix (group 1) the Cys residues are always found in a CXXXC motif.^[26,44] At these sites, the backbone carbonyl oxygen of the first Cys is 2.0 to 2.4 Å from the Cu(I) ion (Figure 2b and Figure 3b), and in some cases can therefore be considered a ligand. There appears to be little organization of group 1 and 2 sites in *MtCsp1*, with the CXXXC-bound Cu(I) ions (group 1) being Cu1, Cu6, Cu8 and Cu12 (Figure 2a). However, in *MtCsp3*, a large part of the Cu(I) core consists largely of alternating group 1 and 2 sites (Figure 3a). In *MtCsp1* all Cys residues bridge between two Cu(I) ions only (μ_2 -S thiolate coordination). Most Cys residues bind in a similar manner in *MtCsp3*, except for Cys101 and Cys111 that exhibit μ_3 -S thiolate coordination. These two Cys residues bind Cu15 along with Cys38, the sole Cu(I)-(S-Cys)₃ site in *MtCsp3*, with Cu4 having the same coordination and distorted trigonal planar geometry in *MtCsp1*, and in both cases all three Cys ligands originate from different α -helices (group 2 sites).

In *MtCsp1*, the only group 3 sites are Cu11 and Cu13, which are bridged by the thioether sulfur of Met48 (Figure 2a, c). These have Cu(I)-(S-Cys)₂(S-Met) and Cu(I)-(S-Cys)(N-His36)(S-Met) coordination respectively, with highly distorted trigonal planar geometries.^[26] *MtCsp3* has a H¹⁰⁴AGNH¹⁰⁸EH¹¹⁰ sequence in the α_3 -loop- α_4 region. His residues corresponding to His104, His108 and His110 are only present in the

Methylocystaceae family of methanotrophs, but are more conserved in non-methanotrophs.^[33] His104 and His110 are ligands to Cu18 and Cu19 (Figure 3a, c) in *MtCsp3*.^[44] Cu19 has Cu(I)–(S-Cys)₂(N-His104) coordination at a distorted trigonal planar site (Figure 3c). His110 binds Cu18 along with the thiolate of Cys111 at a 2-coordinate site with almost linear geometry (Figure 3c). It is possible that another 2-coordinate Cu(I) site involving His108, requiring a 180° rotation of the imidazole ring to enable coordination via its N^δ atom, and Cys42, which would then provide a third μ_3 -S thiolate, could be formed in the presence of an additional Cu(I) ion in *MtCsp3* (Figure 3c),^[44] as in the Csp3 from *S. lividans*.^[45] Two-coordinate Cu(I) bound by Cys and His residues will have a lower Cu(I) affinity than a site with two Cys ligands, and whether these sites are relevant for storage remains to be determined. An exception to the observation that group 3 Cu(I) sites are typically the most solvent-exposed in the Csp3s is provided by Cu13 in *MtCsp3* (Figure 3a), which is relatively far from the mouth of the bundle.^[44] As well as being bound by two Cys thiolates this site is coordinated by the side chain carbonyl oxygen atom of Asn47 (vide infra).

Throughout the cores of both *MtCsp1* and *MtCsp3* there are numerous Cu(I)–Cu(I) interactions.^[26,44] A Cu(I) to Cu(I) distance <2.8 Å is considered to possess some degree of metal-metal bonding as the van der Waals radius of Cu is 1.4 Å.^[52] All but one Cu(I) site in *MtCsp1* is ≤2.8 Å from its nearest neighbour (Cu9 is ~2.9 Å from Cu8).^[26] Four of the 13 Cu(I) ions make two such interactions, with the shortest Cu(I) to Cu(I) distances (2.4–2.5 Å) being at the more solvent-protected end of the bundle between Cu1 and Cu2, and Cu3 and Cu4 (Figure 2a). The greater number of Cu(I) ions accommodated in an *MtCsp3* core of similar size to that of *MtCsp1*, results in more Cu(I)–Cu(I) interactions, and all but one of the 19 Cu(I) ions is ≤2.8 Å from another Cu(I) site, with Cu4 being <2.9 Å from Cu2.^[26,44] The strongest Cu(I)–Cu(I) interactions (~2.4–2.5 Å) are between Cu1 and Cu2, Cu9 and Cu10, Cu13 and Cu14, and also Cu17 and Cu19 (Figure 3a), i.e. are distributed along the bundle. Furthermore, Cu5, Cu7, Cu9, Cu13 and Cu16 are involved in up to three Cu(I)–Cu(I) contacts of ≤2.8 Å. The higher density of Cu(I) ions in *MtCsp3* leads to a more compact arrangement, resulting in Cu3 to Cu14 forming tetranuclear clusters that are key to core formation.^[44,53]

4.3 Core formation in *MtCsp3* and the importance of tetranuclear clusters

The formation of the Cu(I) core in a Csp3 has been visualized by determining crystal structures of *MtCsp3* loaded with increasing amounts of Cu(I) (Figure 4), and is dominated by three tetranuclear clusters involving Cu3 to Cu14 (Figure 5).^[53] In *MtCsp3* binding 1–2 equiv of Cu(I), only one of these is partially occupied; a highly symmetrical [Cu₄(S-Cys)₄(O-Asn)] cluster

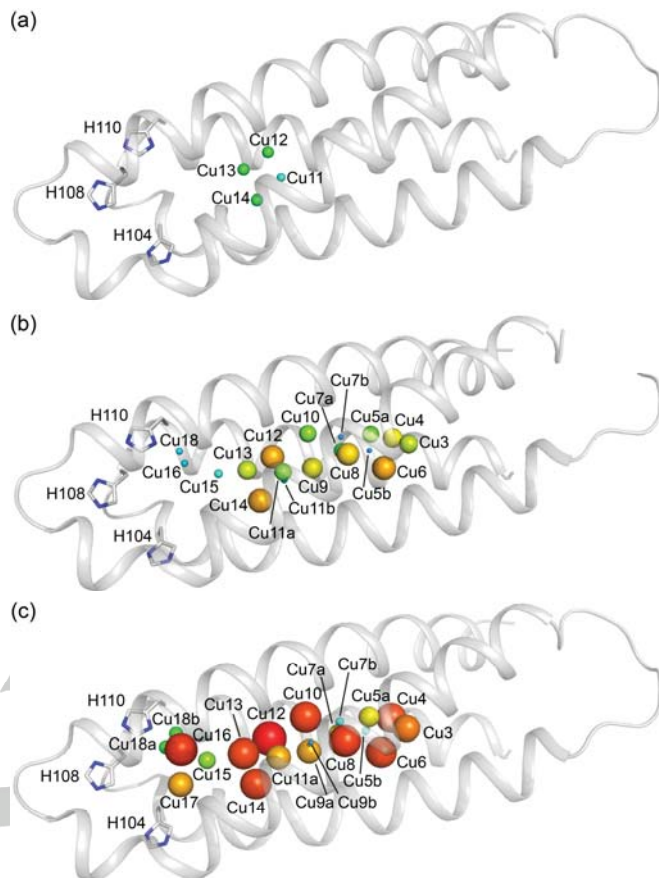


Figure 4. Cu(I) core formation in *MtCsp3*. The structures of an *MtCsp3* monomer, with α_N omitted, binding 1–2 (a, PDB file 5NQM), 8 (b, PDB file 5NQN) and 14 (c, PDB file 5NQO) equiv of Cu(I).^[53] The three His residues at the mouth of the four-helix bundle are shown as sticks. The size and colour (from blue to red for low to high) of the spheres representing the Cu(I) ions indicate relative occupancies of sites. Cu19, only present in the 14 equiv structure, and the minor alternate form of Cu11 (Cu11b), both with occupancies of 0.2 are not visible in (c) as they are obscured by larger spheres (Cu17 and Cu11a respectively).

made up of Cu11 to Cu14 (Figure 4a and Figure 5a). This involves a close to planar rhombus-like arrangement of Cu(I) ions and all μ_2 -S thiolate Cys coordination.^[53] The presence of the side chain carbonyl oxygen of Asn47 coordinating Cu13 (a group 3 site) is an unusual feature of the *MtCsp3* tetranuclear clusters. This residue is not conserved in methanotroph Csp3s, with Met most frequently found,^[33] but is more common in Csp3s from other bacteria, and in some cases, such as *B. subtilis* and *S. lividans*, an Asp is present in this position,^[26,44] which can coordinate.^[45] It seems unlikely that Asn47 plays a role in Cu(I) initially binding at Cu11–Cu14. This probably has more to do with these being the first sites Cu(I) ions encounter when entering *MtCsp3* where tetranuclear cluster formation can occur.^[53]

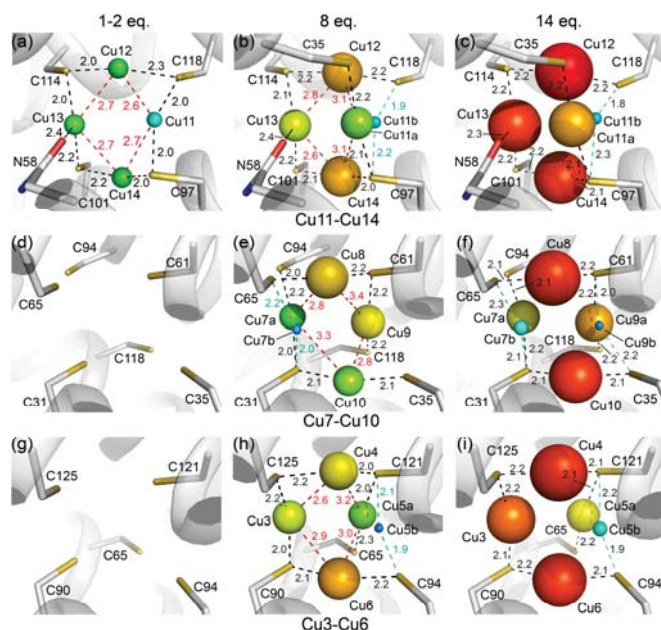


Figure 5. Tetranuclear thiolate-coordinated Cu(I) cluster formation in *MtCsp3*.^[53] The structures of the Cu11-Cu14 (a-c), Cu7-Cu10 (d-f) and Cu3-Cu6 (g-i) clusters in *MtCsp3* binding 1-2 (a, d, g), 8 (b, e, h), and 14 (c, f, i) equiv of Cu(I). The coordinating Cys residues, as well as Asn58 at Cu11-Cu14, are shown as sticks. The size and colour (from blue to red for low to high) of the spheres representing the Cu(I) ions indicate relative occupancies. The Cu-ligand bonds at the major clusters, those at the alternate Cu(I) sites, and Cu(I)-Cu(I) interactions are shown as dashed black, cyan and red lines, respectively, with Cu(I)-ligand bond lengths and Cu(I)-Cu(I) distances in Å.

As *MtCsp3* is loaded with more Cu(I) ions, occupancy at Cu11-Cu14 increases and these sites re-arrange into a less symmetrical $[\text{Cu}_4(\text{S-Cys})_5(\text{O-Asn})]$ cluster (Figure 4 and Figure 5a-c).^[53] Furthermore, two new tetranuclear clusters involving Cu7-Cu10 (Figure 5d-f) and Cu3-Cu6 (Figure 5g-i) are observed, with the major forms involving $[\text{Cu}_4(\text{S-Cys})_6]$ and $[\text{Cu}_4(\text{S-Cys})_5]$ species, respectively. Occupancies increase as more Cu(I) is added to the protein, with total occupancies at the three clusters ranging from 2.4 to 2.7 in the 8 equiv structure and 3.5 to 3.7 in the 14 equiv structure.^[53] Either two (Cu7-Cu10) or three (Cu3-Cu6 and Cu11-Cu14) of the coordinating Cys residues bridge between two Cu(I) ions at the same cluster, whilst the other Cys residues bridge two Cu(I) ions in adjacent clusters (Figure 5), which are also connected by Cu5 to Cu7 (2.7-2.8 Å), and Cu9 to Cu11 (2.6-2.7 Å) interactions. Alternate, and more symmetrical, $[\text{Cu}_4(\mu_2\text{-S-Cys})_4]$ clusters involving minor forms of three of the four group 2 sites at Cu3-Cu6 and Cu7-Cu10 (Cu5b, Cu7b, and Cu9b) are observed (Figure 5f, i), and the initial $[\text{Cu}_4(\text{S-Cys})_4(\text{O-Asn})]$ arrangement in the 1-2 equiv structure includes a minor (alternate) form of Cu11 (Cu11b, also a group 2 site) (Figure 5a-c).^[53] There is flexibility in how the Cys residues from different α -helices can bind Cu(I) at group 2 sites, apart from Cu3 and Cu13 that are situated at the two ends of the three tetranuclear cluster arrangement (Figure 4b, c and Figure 5). This allows the more symmetrical tetranuclear arrangements to occur, which appear to be intermediates in the formation of the final clusters.

Perhaps unexpectedly, the two sites at the hydrophobic end of *MtCsp3* (Cu1 and Cu2) are the last to bind Cu(I), with no sign of occupancy (Figure 4c) in the structure of the protein plus 14 equiv of Cu(I). The filling of these two sites in the structure with 19 equiv of Cu(I) bound causes the position and coordination of Cu3 to change.^[44] The Cu(I) ion at this site is still bound by Cys90 as in the partially-loaded structures (Figure 5h, i), but the side chain of Cys125 undergoes a conformational change and Cys28 replaces it as a ligand at Cu3.^[44,53] This allows Cys125 to coordinate Cu2 along with Cys24, with Cu1 bound by Cys24 and Cys28 (see Figure 3b). The Cu(I) sites at the mouth of the *MtCsp3* bundle start to be observed when the protein is almost half-loaded with Cu(I), and Cu15-Cu19 are all present to varying degrees in the 14 equiv structure (Figure 4b, c).^[53] The most occupied, and only group 1 site, in this location is Cu16, with Cu18 exhibiting alternate forms, and the position of Cu19 shifting as more Cu(I) is added. These sites are bound by five Cys and two His ligands in the structure binding 19 equiv of Cu(I) (Figure 3c).^[44] As mentioned previously, it is possible that an additional Cu(I) site could be coordinated at the mouth of *MtCsp3* by His108 (not a ligand in the 19 equiv structure) and Cys42, resulting in a hexanuclear arrangement.

Crystal structures of partially Cu(I)-loaded forms of *MtCsp1* have not been determined as the Cu(I)-protein is difficult to crystallize. Initial NMR studies of Cu(I) uptake (in collaboration with Prof. Peter Crowley at NUI Galway) indicate that binding is highly fluxional. The lower density of Cys residues, and consequently Cu(I) ions, means tetranuclear clusters are not present in the core of fully-loaded Cu(I)-*MtCsp1*, with the majority of sites contributing to a thiolate-bridged linear structure (Figure 2a).^[26] The Cu10 to Cu13 sites do form a relatively symmetrical, higher nuclearity, arrangement at the mouth of the bundle, which involves three $\mu_2\text{-S}$ Cys ligands, His36 and the bridging thioether sulfur of Met48 (Figure 2c).^[26] Three of the Cu(I)-Cu(I) distances are short (2.7-2.8 Å), whilst that between Cu11 and Cu13 is 3.8 Å. There are similarities between the arrangements at the mouths of Cu(I)-*MtCsp1* and Cu(I)-*MtCsp3* (Figure 2c and Figure 3c).^[26,44] In that they include the main non-Cys ligands and possess only a single group 1 Cu(I) site. Binding at, and the coordination flexibility of, sites at the openings of the bundles, along with the residues present in this region, must be important for the Cu(I) uptake and removal mechanisms of both Csp1s and Csp3s (see Section 4.5).

4.4 Comparison of the structures of copper storage proteins and Cu(I)-binding metallothioneins

The ability of the relatively small Cys-rich metallothioneins (MTs) to bind and store Cu(I) in eukaryotes is well established.^[52,54-59] A Cu(I)-binding homologue (MymT) has been identified in pathogenic mycobacteria^[60] that provided, prior to the discovery of the Csp, a very rare example of Cu storage in bacteria. Eukaryotic MTs are also able to store Zn(II), and can bind other metals with a d^{10} electronic configuration, including the highly toxic Cd(II) and Hg(II).^[56,57,59,61-64] Due to difficulties in obtaining suitable crystals,^[52] NMR has been extensively used to study solution structures of Cu(I)-MTs, but provides limited information

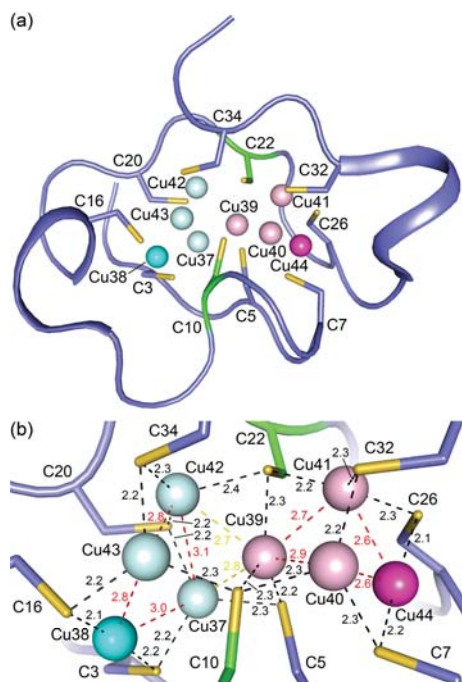


Figure 6. The structure of a Cu(I)-MT. (a) The crystal structure^[52] of a shortened form of *S. cerevisiae* Cu(I)-Cup1 (PDB file 1RJU) with the 10 Cys residues shown as sticks and the eight Cu(I) ions as spheres. (b) Detailed structures of the two interlinked tetranuclear clusters. In both (a) and (b) the Cys residues exhibiting μ_3 -S thiolate coordination (Cys10 and Cys22) are green. Cu37, Cu42 and Cu43 (3-coordinate and pale blue spheres) form a $[\text{Cu}_4(\text{S-Cys})_7]$ cluster with the 2-coordinate Cu38 (cyan). The $[\text{Cu}_4(\text{S-Cys})_6]$ cluster is made up of Cu39, Cu40 and Cu41 (3-coordinate and pale pink) along with the 2-coordinate Cu44 (pink). The Cu–S(Cys) bonds are shown as dashed black lines, the Cu(I)–Cu(I) interactions within a tetranuclear cluster as dashed red lines, and those between clusters as dashed yellow lines, with bond lengths and Cu(I)–Cu(I) distances in Å.

about the Cu(I) sites.^[65–70] The only crystal structures of metallated MTs are for Cu(I)-Cup1 from *Saccharomyces cerevisiae* (Figure 6a)^[52] and rat liver MT-2 binding five Cd(II) and two Zn(II) ions.^[62] In both crystal and NMR solution structures the proteins fold around clusters of metal ions. The Cu(I)-Cup1 crystal structure is of a shortened form with its four N-terminal amino acids and final 13, including two Cys, residues removed, but this does not alter Cu(I) binding^[65,68] as the C-terminus is disordered.^[67] Eight Cu(I) ions are bound by ten Cys residues (Figure 6), resulting in tetranuclear $[\text{Cu}_4(\text{S-Cys})_6]$ and $[\text{Cu}_4(\text{S-Cys})_7]$ clusters, connected by bridging Cys ligands and Cu(I)–Cu(I) interactions (Figure 6b).^[52] The structures of the Cu(I) sites in this MT can be compared to those in Cu(I)-Csp3, and particularly Cu(I)-MtCsp3. The majority (six) of the Cu(I) ions in the Cu(I)-Cup1 structure are 3-coordinate (Figure 6b),^[52] whilst most sites in Cu(I)-MtCsp3, and all in Cu(I)-MtCsp1, are 2-coordinate.^[26,44] The preference for the Cys residues to coordinate as μ_2 -S thiolates is shared in the two families of storage proteins, with only two μ_3 -S thiolates in both Cu(I)-Cup1 (Figure 6b) and Cu(I)-MtCsp3, and none in Cu(I)-MtCsp1.^[26,44,52] The Cu(I) to Cu(I) distances are all similar within the Cu(I)-Cup1 tetranuclear clusters, as is the case for the Cu(I)-MtCsp3

intermediates, but these have a distorted tetrahedral arrangement, like those in the final forms of the Cu(I)-MtCsp3 Cu3–Cu14 clusters (Figure 7).^[44,52,53]

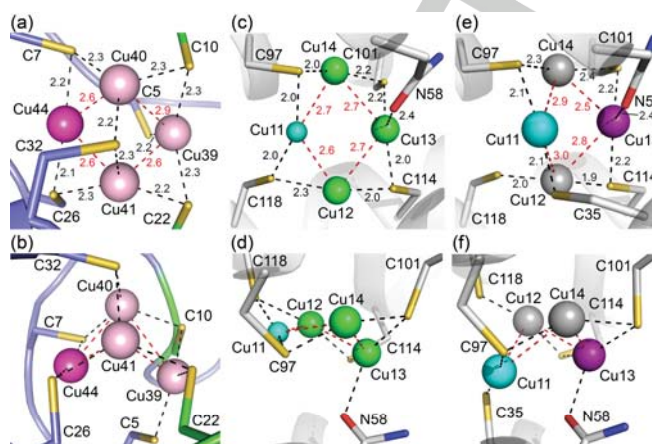


Figure 7. Comparison of tetranuclear Cu(I)-thiolate clusters in an MT and a Csp3. The Cu(I)-Cup1 $[\text{Cu}_4(\text{S-Cys})_6]$ cluster^[52] involving Cu39, Cu40, Cu41 and Cu44 is shown in (a) and (b) in two orientations. For MtCsp3, the intermediate $[\text{Cu}_4(\text{S-Cys})_4(\text{O-Asn})]$ (c and d, 1–2 equiv structure, PDB file 5NQM)^[53] and the final $[\text{Cu}_4(\text{S-Cys})_5(\text{O-Asn})]$ (e and f, 19 equiv structure, PDB file 5ARN)^[44] Cu11–Cu14 clusters are shown. The representation and labelling of the structures is as described in the legends to Figures 3, 5 and 6.

The similar arrangement of Cu(I) sites in Cup1 and MtCsp3, and also presumably other members of these families of proteins, indicates that cluster formation is driven by Cu(I)-thiolate chemistry. This is perhaps to be expected for MTs whose apo-forms are flexible,^[56,63,71] and is probably the key reason why they can bind a range of other metal ions.^[56,57,59,61–64] The ability to bind both Cu(I) and Zn(II) in vivo results in the physiological role of MTs in Cu and Zn homeostasis being intertwined.^[59] The inflexible four-helix bundles of Csp3s, with their Cys-lined cores, seem suited to only Cu(I)-thiolate interactions, making them specific for cuprous ions at the expense of an ability to bind other metal ions. Consistent with this, there is no evidence of Zn(II), or any of the other metal ions that were tested, bound to MtCsp1 when isolated from *M. trichosporium* OB3b, and Zn(II) does not bind tightly to the protein in vitro.^[26,33] As Cu(I)-thiolate chemistry is so versatile the arrangement of the Cys residues and Cu(I) ions within the cores of these proteins can vary. Thus, alternative arrangements to tetranuclear clusters, as seen within the core of MtCsp1, are possible, probably giving rise to different in vitro Cu(I)-binding properties, and maybe also having functional importance, for a Csp.^[33]

4.5 In vitro Cu(I)-binding characteristics of copper storage proteins and metallothioneins

Csp3s and MTs exhibit relatively intense luminescence at ~600 nm when excited within their S(Cys)→Cu(I) ligand-to-metal charge transfer (LMCT) bands.^[44,54,55,57,58,60] Such emission

arises from strong, solvent-protected, Cu(I)-Cu(I) interactions in clusters.^[54,55,60,72] However, *MtCsp1* gives little ~600 nm luminescence upon Cu(I) binding,^[26] which may be due to the absence of higher-order arrangements of Cu(I) ions within its core, as emission is particularly prevalent for tetranuclear and hexanuclear clusters of d¹⁰ metal ions.^[72] This is consistent with *MtCsp3* luminescence reaching a maximum after the protein is partially Cu(I) loaded (9-11 equiv), when the buried Cu3-Cu14 sites that form tetranuclear clusters are most selectively occupied (Figure 4b,c).^[44,53]

How tightly Csps bind Cu(I) has been estimated by determining Cu(I) occupancy as a function of free Cu(I) concentration, buffered by the high-affinity chromophoric ligands BCS and bichinchonic acid (BCA).^[26,44] Fitting data to the Hill equation gives average Cu(I) affinities of $(1-2) \times 10^{17} \text{ M}^{-1}$ for *MtCsp1* and *MtCsp3*, with that for *B. subtilis* Csp3 very similar. Positive cooperativity is found for *MtCsp1*, but cooperativity is absent in any Csp3 studied in detail to date.^[26,44] This is consistent with the preferential binding of Cu(I) at specific sites in the crystal structures of partially Cu(I)-loaded *MtCsp3* (Figure 4), which is probably not observed for *MtCsp1*, and indicates such forms are required for function. Dissimilar Cu(I)-binding mechanisms for *MtCsp1* and *MtCsp3* also supports the idea that these proteins perform different specific storage functions in *M. trichosporium* OB3b.^[33] Whether Cu(I), and other metal ion, binding is cooperative in MTs has been discussed for many years,^[54,55,57,58,63,71,73] but their affinities for Cu(I) have received much less attention. A related approach to that employed for determining the average Cu(I) affinities of the Csps using diethyl-dithio-carbamate, whose Cu(I) affinity was determined indirectly relative to that of dithiothreitol (DTT), to buffer free Cu(I), and monitoring protein metalation with mass spectrometry has been applied to human MTs. This provides an estimated average Cu(I) affinity for MT-2A of $2.4 \times 10^{15} \text{ M}^{-1}$ and positive cooperativity with a Hill coefficient of ~4 (the value for *MtCsp1*^[26] is ~3).^[74] A very similar average Cu(I) affinity has been measured for MT-3 ($2.1 \times 10^{15} \text{ M}^{-1}$), but Cu(I) binding shows weak positive cooperativity (Hill coefficient of 1.3) in this case.^[75] These Cu(I) affinity measurements only considered the 1:1 Cu(I):DTT complex,^[76] as previously discussed,^[77,78] resulting in values being underestimated by as much as 3-4 orders of magnitude. This is highlighted when comparing the Cu(I) affinities obtained for other proteins with this method,^[74] to those measured using BCS and BCA to compete for Cu(I).^[77-79] Therefore, the average Cu(I) affinities of MTs are probably 1-2 orders of magnitude tighter than those of the Csps, consistent with values reported in other studies.^[58,80]

Whilst Cu(I) uptake by all Csps is anticipated to be very fast, potentially diffusion controlled, particularly at the mouths of their bundles, there is a dramatic difference in the rate of Cu(I) removal from Csp1s and Csp3s by BCS.^[26,33,44] Whereas all *MtCsp1*-bound Cu(I) can be extracted using a large excess of this ligand in ~30 min,^[26] only ~20% is removed from *MtCsp3* in 85 h under identical conditions.^[44] Cu(I) removal rates differ for Csp3s, and 85% of Cu(I) is extracted from the *B. subtilis* protein by BCS in 85 h,^[44] and could be even quicker for other Csp3s.

Furthermore, slower Cu(I) removal may occur in *MtCsp1* homologues. However, Cu(I) removal from *Neisseria gonorrhoeae* Csp1 is complete in <3 h. These variations in Cu(I) removal rates are most probably related to the differences in the residues and site structures at the mouths of the four-helix bundles of these proteins as discussed in Section 4.3 (Figure 2a, c and Figure 3a, c), and must be important for their specific functions.^[26,33,44] Cu(I) removal from *MtCsp1* and *MtCsp3* by the apo-Mbn from *M. trichosporium* OB3b is strongly thermodynamically favoured, but is also much faster for Cu(I)-*MtCsp1*^[26] than Cu(I)-*MtCsp3*, and the reaction with the latter is too slow to have importance in the methanotroph.^[44] However, it is unclear if the apo-Mbn interaction with Cu(I)-*MtCsp1* has physiological significance as Mbn is not produced at elevated Cu, whereas the *MtCsp1* gene is upregulated upon switchover in a similar manner to genes encoding pMMO.^[39,81] It is possible that when Cu becomes limiting and switchover from pMMO to sMMO occurs, intracellular apo-Mbn may remove Cu(I) from *MtCsp1* to assist delivery to pMMO, facilitating the prolonged use of this enzyme to oxidise methane.^[26,33] The Cu(I) supplied by Mbn may be stored by a Csp, although direct transfer from Cu(I)-Mbn to an apo-Csp does not occur in vitro due to the significantly higher Cu(I) affinity of Mbn.^[26] The removal rate of Cu(I), and other metal ions, from MTs has also received little attention but appears to be relatively fast,^[56,73] as expected considering the structures of the metallated protein (Figure 6a).^[52,62] Proteolysis and Cys oxidation have been discussed as mechanisms to facilitate metal ion removal from MTs.^[82,83]

5. Copper clusters in other systems

The coordination chemistry of organothiolate Cu(I) complexes is extensive, with varying stoichiometries and structures possible.^[84,85] This is driven by the thiophilic nature of the soft Cu(I) ion, its d¹⁰ electronic configuration that does not impose any electronic preferences on coordination, the ability of Cu(I) to be bound by 2-4 thiolate ligands, and the presence of three lone pairs on the sulfur of a thiolate group. Furthermore, in proteins there is free rotation around the C^β atom of the Cys side chain allowing relocation of its thiolate group to accommodate Cu(I) ions. Collectively, these factors contribute to a number of Cys-containing proteins involved in Cu homeostasis, mainly from the well-studied model system *S. cerevisiae*, binding polynuclear thiolate-coordinated Cu(I) clusters in vitro, with most giving rise to luminescence at ~600 nm. This includes the transcriptional activators Mac1 and Ace1,^[86-88] the mitochondrial Cu(I) metallochaperone Cox17^[89] (also porcine Cox17^[90]), and the Cu(I) importer Ctr1.^[91] A physiological role appears to be established only for the Mac1 and Ace1 clusters.^[86,87,92] No crystal structures of the Cu(I)-proteins have been determined, but most exhibit extended X-ray absorption fine structure (EXAFS) data similar to the [Cu₄(SPh)₆]²⁻ complex,^[88,89,91,93] in which all Cu(I) ions are trigonally coordinated by μ₂-S thiolates.^[94] Similar structures have therefore been predicted in the proteins and must be analogous to the clusters in Cu(I)-Cup1 (Figure 6b), whose EXAFS data is also like that for

$[\text{Cu}_4(\text{SPh})_6]^{2-}$,^[93] as well as to those in $\text{Cu(I)}\text{-MtCsp3}$ (Figure 5). The thiol-containing tripeptide glutathione (GSH) that acts as a ubiquitous intracellular reductant can bind Cu(I) and has been implicated in Cu homeostasis. For example, GSH is required for the activation of Cu,Zn -superoxide dismutase in the absence of the enzyme's dedicated Cu metallochaperone CCS.^[95] The major species formed by Cu(I) and GSH is a tetranuclear $[\text{Cu}_4(\text{GS})_6]$ cluster, also presumed to have a structure similar to $[\text{Cu}_4(\text{SPh})_6]^{2-}$.^[96]

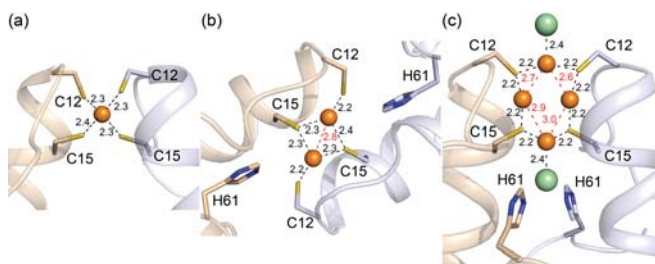


Figure 8. Structures of Cu(I) -Atx1 dimers. (a) The $\text{Cu(I)}\text{-(S-Cys)}_4$ distorted tetrahedral site in the crystallographic dimer formed by $\text{Cu(I)}\text{-ATOX1}$ (PDB file 1FEE).^[100] Also shown are the head-to-head Cu(I) -Atx1 (b, PDB file 2XMT) and $\text{Cu(I)}_2\text{-Atx1}$ side-to-side (c, PDB file 2XMJ) *Synechocystis* PCC 6803 Atx1 dimers.^[102] The Cu(I) ions are represented by orange spheres, with the coordinating Cys residues from the C¹²XXC¹⁵ motifs of both proteins, and the important second-coordination sphere His61 of *Synechocystis* PCC 6803 Atx1, shown as sticks. The two chloride ions at the $[\text{Cu}(\mu_2\text{-S-Cys})_4\text{Cl}_2]^{2-}$ cluster in (c) are represented by light green spheres. The Cu(I) -ligand bonds and Cu(I) - Cu(I) interactions are shown as black and red dashed lines, respectively, with distances in Å.

The structural diversity of Cu(I) -Cys chemistry is further demonstrated by crystal structures of the Atx1 family of cytosolic Cu(I) metallochaperones. These proteins use the thiolates from a CXXC motif to tightly bind^[77,78,91,97] a single, solvent exposed and coordinatively unsaturated, Cu(I) ion that can be readily transferred by ligand exchange.^[98,99] However, there is a tendency for these proteins to form dimers stabilised by intermolecular $\text{Cu(I)}\text{-(S-Cys)}$ bonding, with the arrangement dependent upon the $\text{Cu(I)}\text{:protein}$ ratio. The crystal structure of human ATOX1 has a single Cu(I) ion coordinated by the four Cys residues from the CXXC motifs of two chains (Figure 8a),^[100] either due to insufficient Cu(I) being added to the protein or to Cu(I) dissociating during crystallization. A very similar arrangement is found in crystal structures of the Atx1 from *S. cerevisiae*.^[101] A cyanobacterial Atx1 forms different dimeric species in solution as a function of Cu(I) loading.^[102] These have been crystallized, and the 'head-to-head' dimer that predominates when 1 equiv of Cu(I) is added to the protein binds a dinuclear Cu(I) site with two $\mu_2\text{-S}$, and two terminal thiolates (Figure 8b).^[102] The addition of a second equiv of Cu(I) in the presence of chloride results in a side-to-side dimer that gives rise to weak emission at ~600 nm. In this form the monomers are linked by a symmetrical $[\text{Cu}(\mu_2\text{-S-Cys})_4\text{Cl}_2]^{2-}$ cluster (Figure 8c), with a rhombus-like (almost planar) arrangement of Cu(I) ions, similar to the intermediates that form in the core of *MtCsp3* (Figure 5a and Figure 7c, d).^[53,102] In the

absence of chloride a head-to-head $\text{Cu(I)}_2\text{-Atx1}$ dimer is observed binding a much less symmetrical arrangement of four Cu(I) ions.^[102] His61 is involved in coordinating partially occupied Cu(I) sites at this cluster whose structure is similar to that formed by the Atx1 (CopZ) dimer from *B. subtilis* with four Cu(I) ions bound.^[103] Physiological roles for Cu(I) -Atx1 dimers remain to be established, but they highlight the flexibility of Cu(I) -thiolate coordination chemistry and the types of sites that can readily form in Cys-containing proteins.

Although Cys is very commonly found as a ligand in proteins involved in Cu homeostasis,^[26,44,52,86-88,98,100-104] it is sparingly used to coordinate Cu in enzymes. A Cys ligand is found at the mononuclear 'type 1' Cu sites of cupredoxins that perform intermolecular electron transfer, for example in photosynthesis, and in related domains of redox enzymes.^[105,106] The highest nuclearity Cu site with a Cys ligand in an enzyme is the electron-transferring Cu_A centre, with a $\text{Cu}_2(\mu_2\text{-S-Cys})_2(\text{N-His})_2$ core, found in the cupredoxin-like fold of subunit II of cytochrome c oxidase (COX),^[107,108] and also in nitrous oxide reductase (N_2OR).^[109,110] The rarity of sulfur-coordinated Cu clusters in enzymes, despite their ease of formation, may be due to such arrangements not being able to catalyse physiologically-relevant reactions as they would strongly favour Cu(I) , preventing facile redox chemistry. Consistent with this idea, the tetranuclear Cu_2 site of N_2OR , where N_2 is formed, has seven His ligands and one or two coordinating sulfide anions.^[109,110]

The absence of Cys-bound Cu clusters in enzymes may also be due to difficulties in safely controlling thiolate chemistry and Cu availability. To form the Cu_A site in COX requires a number of proteins both to chaperone and insert the Cu ions, and also to ensure the two Cys residues do not form a disulfide.^[111] If higher nuclearity clusters with additional Cys residues were used, even more complex machinery would be needed. It is emerging that Cu toxicity can involve cuprous ions replacing Fe at Cys bound Fe_4S_4 clusters in certain enzymes,^[112-114] and also blocking Fe_4S_4 cluster assembly.^[114-116] In both cases the mechanisms involved are promoted by the affinity of Cu(I) ions for sulfur ligands. Paradoxically, the same facile cluster chemistry of Cu(I) bound by sulfur ligands that enables safe storage contributes to the cytotoxicity of this metal ion.

6. Summary and outlook

Copper is intimately linked to the ability of methanotrophs to metabolise methane, a potent greenhouse gas. Methanobactins are secreted to sequester Cu under limiting conditions in some methanotrophs. The crystal structures of only four Cu(I) -Mbns have been determined. Further work is needed to elucidate the diversity of these Cu-binding modified peptides, including in non-methanotrophs, and to understand their Cu-dependent production, secretion, re-incorporation and the safe utilization of the Cu they provide. Unsuccessful attempts to decipher the intracellular fate of Cu(I) -Mbns lead to the discovery of Csp in methanotrophs, where they can store Cu(I) for pMMO, and the realization that these proteins are widespread in both methane-

oxidizing, and other, bacteria. Detailed structural information is available for exported and cytosolic Csp, but there is ongoing uncertainty about the active site structure of pMMO, even though it is the key target enzyme for Cu in methanotrophs. Furthermore, a number of other proteins involved in Cu uptake for, and potentially also delivery to, pMMO, as well as those regulating Cu-dependent switchover between pMMO and sMMO, remain to be identified.

The Csp is a tetramer of four-helix bundles that can bind large numbers of mainly thiolate-coordinated Cu(I) ions within their Cys-lined cores. Tetranuclear thiolate-coordinated clusters are formed in MtCsp3 with similar structures to those present in Cu(I)-MTs. Metallothioneins are able to bind a number of other metal ions due to their structural flexibility, which is not possible for the more rigid four-helix bundles of Csp that promote specificity for Cu(I). A lower density of Cys residues, as found in MtCsp1, results in a more linear core arrangement highlighting the adaptability of Cu(I)-thiolate chemistry and its usefulness for Cu(I) storage. The importance of cooperative Cu(I) binding, along with differences in Cu(I) removal rates from Csp, and the factors controlling these, remain to be determined, as do their functional relevance. The physiological role of a Csp has so far only been determined for the exported MtCsp1, which stores Cu(I) for pMMO, and this is another key area requiring further research, particularly for cytosolic Csp3s. The Cu(I) sites in Csp and MTs exhibit similarities to well-characterized organothiolate Cu(I) cluster complexes, and to those that occur in vitro in other Cys-containing Cu homeostasis proteins. Cluster formation involving sulfur ligands is responsible for the emerging toxicity mechanism of Cu(I). Nevertheless, the same chemistry drives the safe storage of Cu(I) in both prokaryotes (Csp) and eukaryotes (MTs) via physiologically-relevant Cys-coordinated biological Cu(I) clusters.

Acknowledgements

This work was supported by the Biotechnology and Biological Sciences Research Council (grant BB/K008439/1), the Natural Environment Research Council (grant NE/F00608X/1), and Newcastle University.

Keywords: Copper • Methanotrophs • Methane oxidation • Methanobactin • Copper storage

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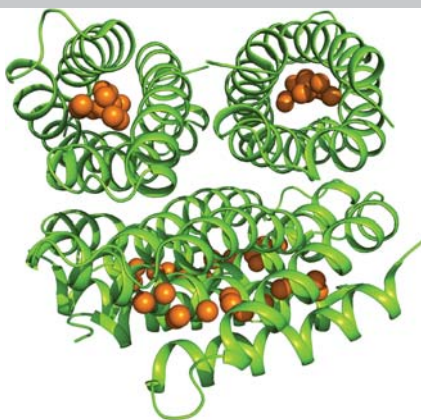
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Entry for the Table of Contents

REVIEW

Methanotrophs utilise large quantities of copper (Cu) to oxidise the potent greenhouse gas methane. To help provide the Cu they require some methanotrophs secrete Cu-sequestering modified peptides called methanobactins. A much greater proportion of methanotrophs possess a member of the newly discovered family of proteins that can store large quantities of Cu for methane oxidation.

*Christopher Dennison***Page No. – Page No.***The Coordination Chemistry of Copper Uptake and Storage for Methane Oxidation**